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Influence of salmonid gill bacteria on development and severity of amoebic gill disease

Sridevi Embar-Gopinath[1,2,*], Rick Butler[1,3], Barbara Nowak[1,2]

[1]School of Aquaculture, University of Tasmania and [2]Aquafin CRC Locked Bag 1370, Launceston, Tasmania 7250, Australia
[3]RSPCA Tasmania, Northern Branch, PO Box 66, Mowbray 7248, Australia

ABSTRACT: The relationship between salmonid gill bacteria and *Neoparamoeba* sp., the aetiological agent of amoebic gill disease (AGD) was determined *in vivo*. Fish were divided into 4 groups and were subjected to following experimental infections: Group 1, amoebae only; Group 2, *Staphylococcus* sp. and amoebae; Group 3, *Winogradskyella* sp. and amoebae; Group 4, no treatment (control). Fish (Groups 1, 2 and 3) were exposed to potassium permanganate to remove the natural gill microflora prior to either bacterial or amoebae exposure. AGD severity was quantified by histological analysis of gill sections to determine the percentage of lesioned filaments and the number of affected lamellae within each lesion. All amoebae infected groups developed AGD, with fish in Group 3 showing significantly more filaments with lesions than other groups. Typically lesion size averaged between 2 to 4 interlamellar units in all AGD infected groups. The results suggest that the ability of *Neoparamoeba* sp. to infect filaments and cause lesions might be enhanced in the presence of *Winogradskyella* sp. The possibility is proposed that the prevalence of more severe AGD is due to the occurrence of *Winogradskyella* sp. at high concentrations on the gills.

KEY WORDS: *Neoparamoeba* · *Winogradskyella* · AGD · Potassium permanganate · Protozoan diseases · Salmon diseases · Amoeba · Bacteria

INTRODUCTION

Amoebic gill disease (AGD) is one of the most significant health problems confronted by the salmon aquaculture industry in Tasmania (Munday et al. 2001). Even though *Neoparamoeba* sp. is presumed to be the causative agent of AGD, the exact environmental conditions or health status of the fish that allow *Neoparamoeba* sp. to proliferate on fish gills are still unknown and Koch's postulate is yet to be fulfilled for the disease (Howard et al. 1993). Until now AGD infection has always been established by cohabiting naïve fish with infected fish (Howard et al. 1993, Akhlaghi et al. 1996, Findlay 2001), or by exposing fish to isolated, gill-associated *Neoparamoeba* sp. (Zilberg et al. 2001, Morrison et al. 2004, Morrison et al. 2005) as the disease cannot be reproduced using cultured organisms (Kent et al. 1988, Howard et al. 1993, Findlay et al. 2000, Morrison et al. 2005).

According to previous studies, AGD outbreaks may be influenced by factors such as predisposing nodules or plaques on the gills, immune status, stocking densities and hyperplastic lesion formation during transfer of salmon from freshwater to seawater (Nowak & Munday 1994, Findlay & Munday 1998, Findlay et al. 2000, Zilberg & Munday 2000, Nowak 2001). Lom & Dyková (1992) also suggest that amphizoic amoebae might typically only colonize the gills of partially immunosuppressed fish where bacterial growth and mucus provide a ready food source. Furthermore, Bowman & Nowak (2004) identified a series of bacteria representing a range of distinct ecotypes from the gills of healthy and AGD infected marine farmed Atlantic salmon. These authors suggested that gill bacteria might play a direct role by predisposing the fish to AGD, to exacerbate AGD, or if bacteria are present in increased numbers in water, might be coincident with AGD outbreaks (Bowman & Nowak 2004). Therefore, the aim of this research is to determine the role of some previously identified salmonid gill bacteria in the incidence and severity of AGD.

*Email: sembar@utas.edu.au

MATERIALS AND METHODS

Fish. Atlantic salmon *Salmo salar* L. (n = 72; mean weight = 88 g) were acclimatised to sea water (35‰, 1 µm filtered) over a week in 6 identical recirculating systems each consisting of three 70 l tanks (n = 4 fish per tank) and a 70 l reservoir. A sentinel population (n = 12) of the same body weight was acclimatised in a static tank (210 l). Following acclimatisation, fish in the recirculating systems were divided into 3 treatment groups (n = 12 fish per treatment). Each treatment was duplicated. The 4th group was the sentinel population (n = 12). Fish in Group 1 were exposed to amoebae only (positive control); Group 2, Gram positive bacteria (*Staphylococcus* sp.) and amoebae; Group 3, Gram negative bacteria (*Winogradskyella* sp.) and amoebae; Group 4 did not receive any treatment. Sea water temperature was maintained at 16 ± 0.5°C, pH 8.2, dissolved oxygen 7.6 mg l^{-1}, salinity 35‰ and total ammonia-nitrogen below 0.2 mg l^{-1}. Sufficient air supply was maintained in the tanks throughout the experiment by using aerators.

***Neoparamoeba* sp. isolation.** *Neoparamoeba* sp. were harvested from the AGD affected Atlantic salmon held in the Aquaculture Centre, University of Tasmania, Launceston by a method described by Morrison et al. (2004). In brief, infected gills were removed from AGD affected Atlantic salmon after euthanasia (anaesthetic overdose at 20 ml l^{-1}Aqui-S®). Gills were transported to the laboratory in sterile sea water (SS) containing antibiotic and antimycotic solution (5% v/v 5000 IU ml^{-1} penicillin and 5 mg ml^{-1} streptomycin solution (Sigma), 1% v/v 10 mg ml^{-1} gentamycin (Sigma) and 0.25 mg ml^{-1} amphotericin B (Invitrogen). The gill arches were separated and scraped to remove the amoebae. Gill arches were then placed in sterile distilled water to loosen the rest of the attached amoebae and the mixture was centrifuged at 400 × *g* for 5 min. The supernatant was discarded and the pellets were resuspended in SS and diluted approximately 50 fold, lightly agitated and decanted into several Petri dishes. Amoebae were left to adhere to the bottom of the Petri dish for 1 h, then the liquid transferred into new Petri dishes to allow adherence of any remaining amoebae for another hour. The fluid was then removed and the Petri dishes were washed several times with SS to remove mucus and epithelial cells, while the amoebae remained attached to the bottom of the Petri dish. The amoebae were detached by adding 750 µl trypsin-EDTA solution (0.025% trypsin per 1 mM EDTA; Invitrogen) and by gently tapping the Petri dishes for a minute. The suspension was then pooled and diluted with SS and centrifuged at 400 × *g* for 10 min. The pellets were resuspended in SS and the amoebae were counted using a trypan blue exclusion assay (Phillips 1973) and haemocytometer to give the number of viable cells in solution. Lack of culturable bacteria in the amoebae inoculum was confirmed by plating the inoculum on marine Sheih's agar and Zobell's marine agar at 25°C for 48 h.

Bacteria. *Staphylococcus* sp. (Gram positive) and *Winogradskyella* sp. (Gram negative) bacteria were selected from previously isolated and characterised gill bacteria strains from AGD affected Atlantic salmon from commercial farms in Tasmania. *Winogradskyella* sp. bacteria were cultured in Sheih's broth (Song et al. 1988) and *Staphylococcus* sp. bacteria were cultured in Todd Hewitt broth (Oxoid). Both were incubated at 22°C for 24 to 48 h. The colony morphology and biochemical profiles (API 50 CH and API Zym, BioMérieux Australia Pty.) of *Winogradskyella* sp. and *Staphylococcus* sp. were noted for identification purposes. Briefly, *Winogradskyella* sp. were Gram negative, rod shaped cells, with a cell length of 0.86 µm and cell width 0.39 µm. The colonies were yellow pigmented, entire and translucent with low convex elevation and 2 mm in length. The API 50 CH test showed that *Winogradskyella* sp. formed acid with glycerol, L-arabinose, ribose, D-xylose, galactose, glucose, mannose, manitol, sorbitol, melibiose, sacarose, trehalose, D-fucose, D-arabitol, L-arabitol and gluconate. The API Zym test showed that *Winogradskyella* sp. metabolised alkaline phosphatase, esterase lipase (C8), α-chymotrypsin, leucine arylamidase, valine arylamidase, acid phosphatase and napthol-AS Bl phosphohydrolase. *Staphylococcus* sp. were Gram positive, coccus shaped cells arranged in clusters. The cell length was 0.9 µm and width was 0.39 µm. The colonies were yellow pigmented, entire and opaque with low convex elevation and 2 mm in length. *Staphylococcus* sp. formed acid with glucose, fructose, manitol, maltose and sacarose. It also metabolised esterase (C4) and napthol-AS Bl phosphohydrolase.

Inoculation with bacteria and amoebae. Prior to inoculation, fish from the treatment groups were transferred using dip-nets from recirculation tanks to individual static tanks for a short term bath (20 min) in sea water (208 l) containing potassium permanganate (5 mg $KMnO_4$ l^{-1}) to remove the natural microflora on the gills (Jee & Plumb 1981, R. Francis-Floyd & R. Klinger, available at http://edis.ifas.ufl.edu/FA027). The sentinel fish were handled in the same way but were bathed in a tank containing sea water only. After the bath, fish were transferred back to their respective systems and were maintained for 2 d to return to normal conditions. To establish baseline community structure, 2 fish from each group were euthanised as described previously and gill mucus and kidney samples were collected and inoculated onto a range of media including Sheih's medium (Song et al. 1988),

Marine Agar (Difco), Tryptone Soya Agar (Oxoid), Todd Hewitt (Oxoid). The agar plates were incubated at 22°C for 24 to 48 h. The remaining fish in Groups 2 and 3 were inoculated with *Staphylococcus* sp. and *Winogradskyella* sp. respectively by transferring fish using individual nets into 2 static tanks (208 l) containing either *Staphylococcus* sp. or *Winogradskyella* sp. bacterium at a density of 1×10^8 cells l^{-1} and bathed for 1 h. Groups 1 and 4 were handled in the same manner but were bathed in sea water only. After transferring back to their respective systems, fish were maintained for 4 d to allow the development of inoculated colonies on the gills. All the groups, with the exception of Group 4 were then infected with *Neoparamoeba* sp. at a concentration of 300 cells l^{-1}.

Sampling procedures. The experiment was terminated and fish from all groups sampled on Day 8 post-amoebae challenge when white mucous patches (consistent with AGD gross pathology) were observed on the gills of the treatment groups. Fish were euthanised as described above and swabs of gill mucus and anterior kidney were taken and inoculated onto bacterial media (listed above) then incubated at 22°C for 48 h. Colonies were then examined and confirmed as either *Staphylococcus* sp. (Group 2) or *Winogradskyella* sp. (Group 3) by comparing the morphology (shape, pigmentation colour, size and appearance on culture plates) and the biochemical profiles with the previously recorded data.

Immediately after swabbing for bacteriology, all gills were removed and placed in sea water Davidson's fixative and post-fixed in 70% ethanol. Individual arches were removed and processed for routine wax histology and stained with haematoxylin and eosin. To visualise Gram positive or negative bacteria on the gills, Group 2 and 3 sections were also Gram stained. All sections were viewed by a light microscope (Olympus) at 4–1000× and fish with typical AGD lesions characterised by a single or multifocal epithelial hyperplasia of the gill lamellae, focal fusion of secondary lamellae and round to ovate interlamellar vesicles containing amoeba were considered AGD positive. Quantitative analysis of disease severity was conducted by estimating the number and size of AGD lesion on each filament (Adams & Nowak 2001). The percentage of lesioned filaments was determined by estimating the ratio of filaments with typical AGD lesions to filaments with no AGD lesions. Lesion size was analysed by counting the number of hyperplastic interlamellar units within each lesion. Images of the affected tissue were taken using a Leica DC300f digital camera (Wetzlar).

Statistical analysis. A 1-way ANOVA using SPSS© version 11.5 was performed to test for significant difference between the experimental groups. Significant differences between groups were assessed using Tukey's HSD post hoc test. The homogeneity of variance was assessed using Levene's test of equality of error variances. Differences were considered significant at the $p < 0.05$ level.

RESULTS

All groups infected with gill amoebae showed gross gill lesions by Day 8 of the experiment. The negative control (Group 4) did not have gill pathology consistent with AGD at any time during the experiment.

Histopathology

All amoebae infected fish (Groups 1, 2 and 3) had AGD. However, fish exposed to *Winogradskyella* sp. (Gram negative bacterium; Group 3) had significantly more filaments (51%) with lesions than the other groups ($F = 21.9$, df = 3,73, $p < 0.001$; Fig. 1). The majority of histological sections in this group showed the presence of large numbers of Gram negative bacteria on the filaments (Fig. 2A) whereas in Group 2, (pre-exposed to *Staphylococcus* sp.) only small numbers of bacteria were observed. There was no difference in percentage of affected filaments in Group 2 compared to Group 1 (no bacteria); both groups of fish had approximately 16% of gill filaments with lesions. Despite the significant increase in the percentage of

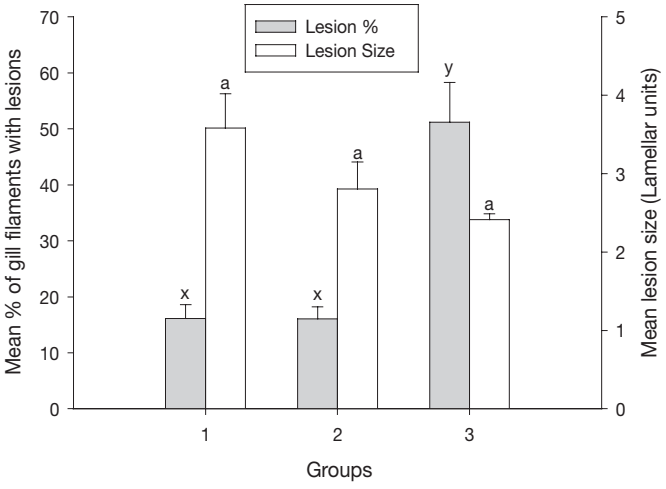

Fig. 1. *Salmo salar*. The mean + SE of percentage of filaments with lesions and number of infected lamellae within in each lesion in experimental groups: (Group 1, *Neoparamoeba* sp.; Group 2, *Staphylococcus* sp. & *Neoparamoeba* sp.; Group 3, *Winogradskyella* sp. & *Neoparamoeba* sp.). Separate analyses were done for lesion percentage and lesion size (lamellar units). Different letters above error bars indicate groups are significantly different. n = 22 for all treatment groups

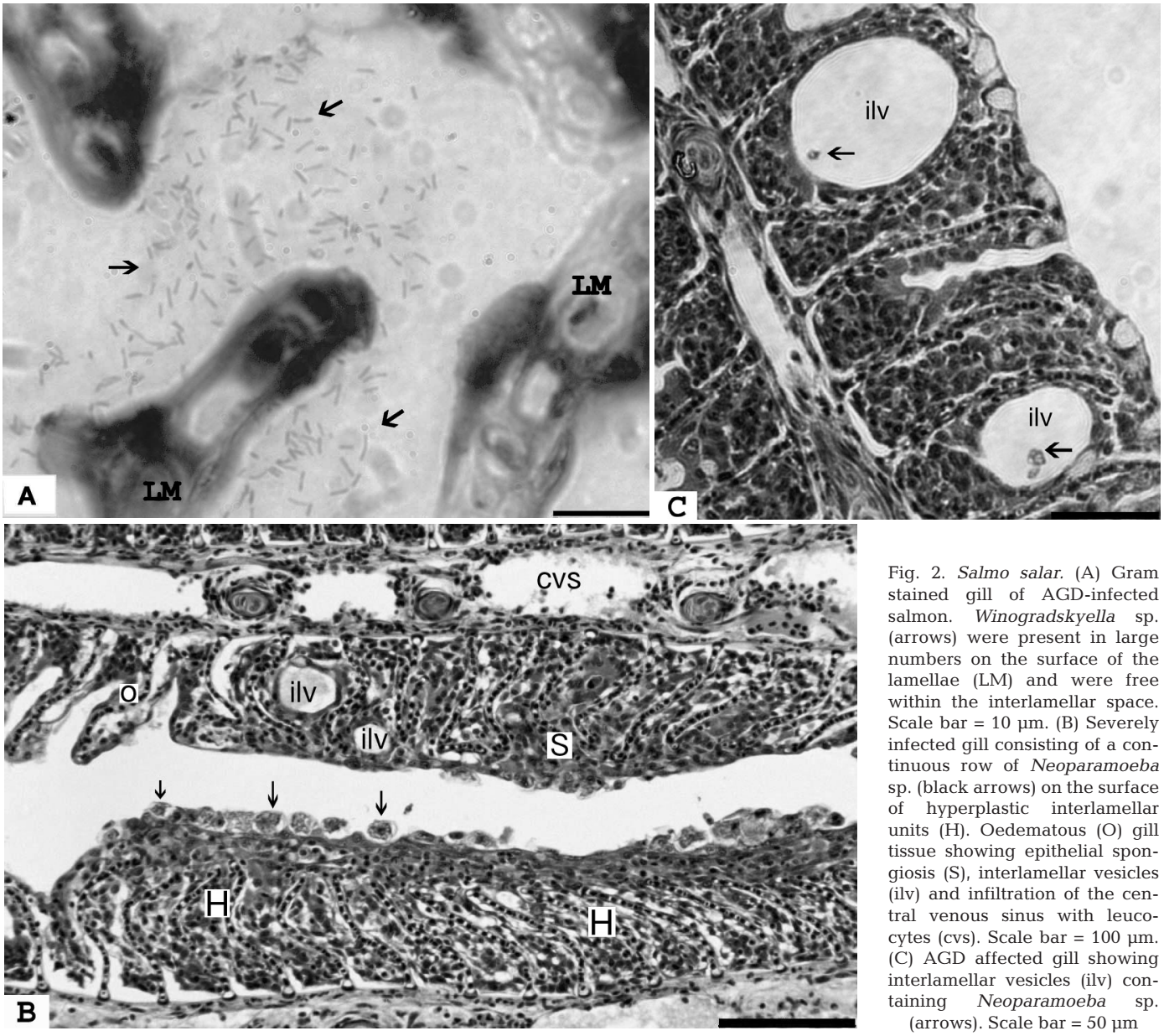

Fig. 2. *Salmo salar.* (A) Gram stained gill of AGD-infected salmon. *Winogradskyella* sp. (arrows) were present in large numbers on the surface of the lamellae (LM) and were free within the interlamellar space. Scale bar = 10 µm. (B) Severely infected gill consisting of a continuous row of *Neoparamoeba* sp. (black arrows) on the surface of hyperplastic interlamellar units (H). Oedematous (O) gill tissue showing epithelial spongiosis (S), interlamellar vesicles (ilv) and infiltration of the central venous sinus with leucocytes (cvs). Scale bar = 100 µm. (C) AGD affected gill showing interlamellar vesicles (ilv) containing *Neoparamoeba* sp. (arrows). Scale bar = 50 µm

lesions on each filament in Group 3, there was no difference in the size of lesions (interlamellar units) between treatment groups. Typically lesions size averaged between 2 to 4 interlamellar units (Fig. 1). In addition, there were no apparent differences in lesion structure. A thorough investigation of the gill sections revealed that all the treatment groups had severe lesions consisting of completely fused secondary lamellae and an almost continuous layer of amoebae was observed on the surface of hypertrophic tissue. Lesions were typified by epithelial desquamation, filamental and lamellar oedema, interlamellar vesicles containing amoebae, infiltration of the central venous sinus with leucocytes and severe hyperplasia of the epithelium (Fig. 2B,C). The control group had healthy gills free from AGD infection. In all cases there were no significant differences between replicates within treatments.

Bacterial recovery

No bacterial growth was detected on the culture plates from the gill and kidney swabs collected from fish 2 d post-$KMnO_4$ bath. At Day 8 post-amoebae infection gill bacteria were very low in number, absent or non-culturable from the gills of Groups 1 and 4 and from the kidney of all the groups. Inoculated bacteria were recovered from the fish gills in Groups 2 and 3

on Day 8 post-amoebae infection. In each case the majority of the recovered colonies were of a single species with morphological and biochemical characteristics consistent with previously recorded data of *Staphylococcus* sp. (Group 2) and *Winogradskyella* sp. (Group 3).

DISCUSSION

Roubal et al. (1989) were the first to suggest a role for gill colonising bacteria in AGD and considered that management strategies aimed at reducing bacterial levels could in turn reduce AGD. Similarly Bowman & Nowak (2004) provided discussion on the presence of *Neoparamoeba* sp. and high bacterial populations that may lead to more pronounced incidence of AGD. The present study is the first experimental attempt to determine a relationship between some gill-associated bacteria and AGD.

The study showed an apparent involvement of the Gram negative bacterium *Winogradskyella* sp. in AGD. Fish experimentally infected with this bacterium showed increased numbers of gill lesions following *Neoparamoeba* sp. infection, whilst those exposed to Gram positive *Staphylococcus* sp. did not show gill pathology that was different from the positive control (Group 1). Positive control animals were previously treated with $KMnO_4$ and gill bacteria were very low in number, absent or non-culturable during subsequent AGD infection. These fish did however develop the disease at a rate comparable to those infected with *Staphylococcus* sp. and consistent with the typical pattern of AGD initiated by experimental infection. All fish for this study were from the same source and same handling procedures were followed for all groups during the entire period of the experiment except for the treatment. Therefore, despite the fact that the gill bacterial status at the beginning of the infection is unknown, the difference in AGD lesion severity between the treatment groups is exclusively due to experimental addition of bacteria.

Fish gills are in continuous contact with the bacteria present in the aquatic environment and consequently some of the bacteria may be trapped on gill filaments and may colonise and become incorporated into the resident microflora (Bowman & Nowak 2004). Roubal et al. (1989) observed the association of bacteria with amoebae during infection of salmon gills. Similarly, Douglas-Helders et al. (2003) reported a co-existing *Flavobacterium* infection on AGD infected salmon gills. Bowman & Nowak (2004) showed a higher proportion of Gram negative bacteria on the gills of marine farmed AGD infected Atlantic salmon. In the present study, a high percentage of hyperplastic lesions on each filament were noted on the gills in the presence of the Gram negative bacterium *Winogradskyella* sp. Phylogenetic analysis of 16S rDNA sequences revealed the nearest neighbour of *Winogradskyella* sp. as *Psychroserpens burtonensis* with a 93.5 to 93.8% similarity (Nedashkovskaya et al. 2004). Further corroborating evidence by Bowman & Nowak (2004) indicated that a *Psychroserpens* sp. phylotype dominated the bacterial community in AGD infected salmon gill samples.

The substrate for *Neoparamoeba* sp. survival and growth, once it has gained access to salmon gills, has not been clearly identified. Previous researchers suggest that other infectious amoebae may feed on gill bacteria (Noble et al. 1997) and perhaps attain bloom populations in the presence of abundant food organisms (Kent et al. 1988). *In vitro* growth of trophozoites of a *Platyamoeba* strain isolated from the diseased gill tissues of cultured turbot increased considerably in the presence of *Aeromonas hydrophila*, *Vibrio natriegens*, *Pseudomonas nautica* and *Escherichia coli* (Paniagua et al. 2001). Similarly, the number of *Acanthamoeba castellanii* and *A. polyphaga* were enhanced by co-cultivation with the Gram negative bacteria, *Xanthomonas maltophilia*, *Flavobacterium breve* and *Pseudomonas paucimobilis* (Bottone et al. 1992). Based on the above findings, it is tempting to suggest that bacteria might provide a food source to *Neoparamoeba* sp. during initial stages of colonisation and infection. However, recent research on AGD has suggested that some cultured strains of *Neoparamoeba* sp. isolated from AGD infected fish gills are not bacterivorous (Dyková & Lom 2004). Despite this, during the present study the fish inoculated with *Winogradskyella* sp. and amoebae showed an increased number of gill lesions. Therefore, some interaction exists between bacteria and amoebae which needs to be characterised. Bracha & Mirelman (1984) reported that co-incubation of *Entamoeba histolytica* with various types of Gram-negative bacteria increased the virulence and ability of amoebae to destroy monolayers of baby hamster kidney cultured cells. Therefore, one explanation for the increased number of AGD lesions noted in this study might be the presence of *Winogradskyella* sp. on the gills which possibly enhanced the ability of *Neoparamoeba* sp. to infect filaments and cause AGD lesions.

The current study also showed that *Neoparamoeba* sp. can infect gills and cause AGD in salmonids (Group 1) after $KMnO_4$ disinfection and with very low levels of culturable bacteria. Therefore, in agreement with other authors we have shown that the amoeba can be a primary pathogen and cause AGD in salmon (Kent et al. 1988, Roubal et al. 1989, Munday et al. 1990, Dyková et al. 1995, Zilberg & Munday 2000, Adams & Nowak 2003, 2004).

We propose that the ability of *Neoparamoeba* sp. to infect filaments and cause lesions might be enhanced in the presence of *Winogradskyella* sp. However, further research is needed to determine whether an increase in the concentration of *Winogradskyella* sp. on the gills increases the rate of incidence and severity of AGD. The effect of *Winogradskyella* sp. alone on the fish gills should also be determined. In addition, it is essential to find the effect of other gill-colonising organisms on AGD. If bacteria are found to influence the progression and severity of AGD then the knowledge may be useful for designing alternative control strategies.

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